# An Active Antibody Fragment (Fv) Composed of the Variable Portions of Heavy and Light Chains†

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ABSTRACT: An active antibody fragment of molecular weight 25,000 was prepared in high yield by pepsin digestion of protein 315, a mouse IgA myeloma protein possessing *anti*-2,4-dinitrophenyl activity. This fragment, denoted Fv, is the N-terminal half of the Fab fragment and is composed of two peptide chains held together by noncovalent bonds. These peptide chains were separated by chromatography on DEAE-cellulose in 8 M urea. The molecular weight of each of the chains in 5.7 M guanidine hydrochloride was found to be 12,500. The separated chains were identified by N-terminal analysis and partial sequence and amino acid composition as the variable portions of the light and heavy chains ($V_L$ and $V_H$) and they contain 110–120 residues each. Amino acid composition also suggests that the position of the pepsin split in the L chain is around residue 115. The separated, inactive $V_L$ and $V_H$ can readily recombine, upon mixing in an equimolar ratio, to yield an active fragment.

The amino acid sequences of immunoglobulins[1] indicate some general features of the gross architecture of these molecules. (1) Each chain is comprised of homology regions, 110–120 residues long. The light chain has two such regions and the heavy chain four. The N-terminal region of each chain is the variable portion, whose sequence varies from one molecule to another, whereas the rest of the sequence is identical within subclass. (2) The intrachain disulfide bonds are linearly and periodically distributed in the structure such that each homology region includes one disulfide bond. This leads to the hypothesis that each homology region folds independently into a compact domain stabilized by one disulfide bond and linked to the neighboring domains by less tightly folded stretches of the peptide's chain (Edelman, 1971). Another consequence of this arrangement is that noncovalent interactions take place between two identical or homologous domains on different peptide chains, to yield a globular unit which performs a specific function.

This general arrangement of the molecule (Figure 1) finds support in recent X-ray crystallographic studies of the Fab′ fragment (Poljak *et al.*, 1972) and the light chain (Epp *et al.*, 1972). Moreover, it is possible, under controlled conditions, to split the molecule between different globular units or domains. The most common place for such splits is the hinge region, where various enzymes or CNBr cleaves the immunoglobulins to Fab and Fc (for summary see Givol and DeLorenzo, 1968). Enzymic splits between neighboring domains of the light chain, yielding intact $V_L$ and $C_L$, were also reported (Solomon and McLaughlin, 1969; Karlsson *et al.*, 1969). More recently, specific cleavage between the globular units of the heavy chain ($C_H2$ and $C_H3$) in the Fc region was reported. This can be achieved either on the intact molecule (Connell and Porter, 1971) or on the isolated Fc fragment (Ellerson *et al.*, 1972). Digestion of the Fab with subtilisin resulted in the recovery of one major fragment, predominantly composed of $C_L$ (Einergall *et al.*, 1972).

We have demonstrated that the Fab′ of protein 315 (mouse IgA myeloma protein possessing anti-Dnp activity, Eisen *et al.*, 1968) can be digested by pepsin to yield a fragment which is half the size of Fab′ and retains all the binding activity of the intact protein (Inbar *et al.*, 1972). This fragment (denoted Fv) is the N-terminal fragment of Fab′ and contains the variable portions of both heavy and light chains. Its size may, therefore, be the minimal possible size for a fragment which possesses an antibody site. This finding also implies that the constant portions of Fab do not contribute to antigen binding.

In the present work we report further details on the preparation and characterization of Fv from protein 315, the separation and identification of its peptide chains ($V_L$ and $V_H$, see Figure 1), and the successful recombination of the isolated chains to yield an active Fv fragment.

## Materials and Methods

*Preparation of Fv.* Protein 315 and its pepsin-produced Fab′ fragment were prepared as described (Inbar *et al.*, 1971). The Fab′ fragment (10 mg/ml in 0.15 M NaCl–0.01 M sodium phosphate, pH 7.4) was brought to pH 3.8 by the addition of 1 M sodium acetate, pH 3.7 ($^1/_{10}$ of the total volume). To the turbid protein solution, pepsin (10 mg/ml in 0.01 M sodium acetate, pH 3.7) was added to give a weight ratio of 1:100 of enzyme to Fab′. After 4 hr at 37° the digestion was terminated by adjusting the pH to 7.0 with 2 M Tris-HCl, pH 8.2. Most of the precipitate dissolved upon raising the pH, and the remaining precipitate, less than 5% of the original Fab′, was removed by centrifugation. The supernatant was applied to a Dnp-lysine Sepharose column equilibrated and run with 0.05 M NaCl–0.003 M sodium phosphate, pH 7.4. After washing the unadsorbed fraction, the column was eluted with Dnp-glycine (0.05 M, pH 7.4) and the yellow fraction was collected, concentrated by vacuum dialysis, and applied to a Sephadex G-75 column, to separate Fv from undigested Fab′ (Inbar *et al.*, 1972). In the affinity chromatography step, 1 ml of Dnp-lysine Sepharose column was used per 2 mg of digest and 0.3 ml of Dnp-glycine was used for elution. The Fv fragment was also prepared directly from protein 315 monomer without the prior isolation of Fab′.

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[1] The nomenclature is in accord with that suggested by the Conference on Nomenclature for Animal Immunoglobulins, Prague, June 1969. See also *Biochemistry 11*, 3311 (1972). Other abbreviations are: Dnp, 2,4-dinitrophenyl; PCA, pyrrolidonecarboxylic acid residue.

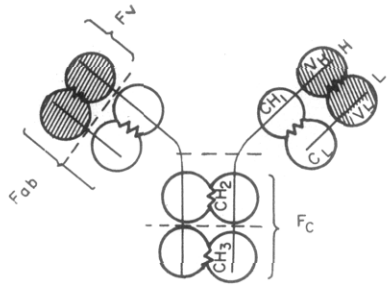


FIGURE 1: Schematic representation of immunoglobulin molecule showing domains and their interactions. Adapted from Edelman and Gall (1969). Dashed lines indicate the positions of various splits.

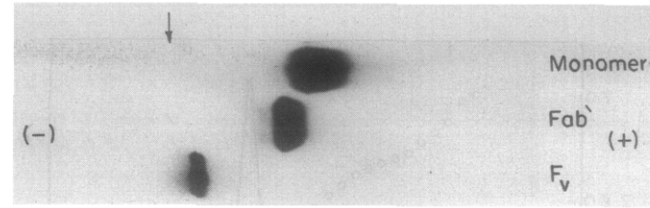


FIGURE 2: Cellulose acetate electrophoresis of protein 315 monomer, Fab′, and Fv. Electrophoresis was for 1 hr at 225 V. Arrow indicates point of application.

*Physical Measurements.* Protein concentration was evaluated from absorbance measurement at 280 nm with a Zeiss PMQ II spectrophotometer. The extinction coefficient of a 1% solution of Fab′ and protein 315 was taken as 14.0 (Eisen *et al.*, 1968). For Fv, an extinction coefficient of 15.0 was determined by nitrogen analysis assuming a nitrogen content of 15%. Fluorescence measurements were determined with a Turner Model 210 recording spectrofluorometer.

Isoelectric focusing was performed with LKB 8100 ampholine electrofocusing equipment using a 110-ml column and pH gradient between pH 5.0 and 7.0, according to the producer manual. Disc gel electrophoresis, was carried out in neutral 1% sodium dodecyl sulfate in 10% polyacrylamide (Shapiro *et al.*, 1967). Cellulose acetate electrophoresis was performed in a Microzone electrophoresis cell (Model R-101) (Beckman-Spinco) using either Beckman B-2 buffer (ionic strength = 0.075) or 0.1 M Tris-HCl, pH 9.0, in 8 M urea. Protein was fixed and stained with Ponceau S staining solution.

Ion exchange chromatography, to separate the peptide chains of Fv, was performed on DEAE-cellulose (Whatman DE-32) in 8 M urea. The urea solution (9 M) was deionized, before use, on amberlite MB-3 (British Drug House) and the conductivity of the deionized solution was below 10 μmho. This urea solution was brought to the desired pH and salt concentration by the addition of the appropriate concentrated buffer solutions and NaCl.

Molecular weights were determined by sedimentation equilibrium in a Beckman Model E ultracentrifuge equipped with a photoelectric speed control and a double scanner. Samples were run in a double sector Epon cell and the temperature was controlled by RTIC. The partial specific volume ($\bar{v}$) was calculated from amino acid composition (Cohn and Edsall, 1943). The sedimentation equilibrium of Fv was performed in 0.15 M NaCl–0.01 M sodium phosphate, pH 7.4. The $\bar{v}$ used for Fv was 0.723. The initial protein concentration was 0.27 mg/ml and the speed was 20,000 rpm. The sedimentation equilibrium of $V_L$ and $V_H$ was in 5.7 M Gdn·HCl. In calculating the molecular weights, $\bar{v}$ values lower by 0.02 $cm^3/g$ than those calculated, were used (Kielley and Harrington, 1960). The values of $\bar{v}$ for $V_H$ and $V_L$ were 0.696 and 0.710, respectively. The density used for 5.7 M Gdn·HCl was 1.155. Initial protein concentrations were 0.4 and 0.2 mg/ml for $V_L$ and $V_H$, respectively. The speed was 30,000 rpm.

*Chemical Analyses.* Amino acid analyses were performed on a Beckman Model 120B amino acid analyzer. Protein samples were hydrolyzed in evacuated sealed tubes with 6 N HCl at 110° for 24 hr. The tryptophan content was estimated from absorbance measurements according to Edelhoch (1967). N-Terminal residues were determined by dansylation in 8 M urea (Gray, 1967) and blocked pyrrolidonecarboxyl residue peptides were obtained and analyzed according to Wilkinson *et al.* (1966) and Rude and Givol (1968). The N-terminal sequence of Fv was performed on a Beckman sequencer according to Wang *et al.* (1970). The phenylthiohydantoin derivatives of amino acids were hydrolyzed with 6 N HCl at 150° for 20 hr and the recovered amino acids were determined on the amino acid analyzer.

*Binding Measurements.* The binding activity of proteins to Dnp-lysine was assessed by quenching of protein tryptophan fluorescence with Dnp-lysine (Eisen 1964).

*Materials.* Dialysis bags were acetylated to reduce pore size according to Craig and Konigsberg (1961). The acetylated bags were used only for dialysis of the peptide chains of Fv. Gdn·HCl, ultra pure, was purchased from Schwarz–Mann.

## Results

*Preparation of Fv Fragment.* The disappearance of Fab′ and appearance of Fv during pepsin digestion can be followed in the Fab′ digest by cellulose acetate electrophoresis. As shown in Figure 2, Fv differs markedly from Fab′ in its electrophoretic mobility. We found that after 4 hr of digestion the only stained band on the cellulose acetate was that of Fv. Affinity chromatography of the neutralized, 4-hr digest, on Dnp-lysine Sepharose, yielded 50% of the applied $A_{280}$ as unadsorbed fraction, whereas 48% of the applied $A_{280}$ was adsorbed and eluted with Dnp-glycine. When this material was chromatographed on a Sephadex G-75 column, 90% of the $A_{280}$ absorbance emerged from the column later than Fab′, in the position of the Fv fragment (Inbar *et al.*, 1972). If protein 315 monomer was digested with pepsin at pH 3.8, the yield of Fv was lower. On the other hand, a good yield of Fv can be obtained by digesting protein 315 monomer with pepsin, at pH 4.5, for 6 hr (Inbar *et al.*, 1971), followed by adjusting the solution to pH 3.8, addition of pepsin (1:100 w/w), and further incubation for 4 hr. Fv can be isolated from this solution by affinity chromatography with a yield of 24% of the $A_{280}$ of protein 315 monomer. We now use this method routinely for the preparation of Fv.

*Characterization of Fv.* The isoelectric focusing pattern of Fv (Figure 3) demonstrates that it is a homogeneous protein with an isoelectric point at pH 5.8.

The molecular weight of Fv was determined by sedimentation equilibrium, using a plot of the logarithm of concentration *vs.* the square of the distance from the axis of rotation, in this experiment. The molecular weight of Fv, calculated from the slope using $\bar{v}$ of 0.723, was found to be 25,250 ± 1200. This result is lower than that previously determined (Inbar *et al.*, 1972) from sedimentation and diffusion data,

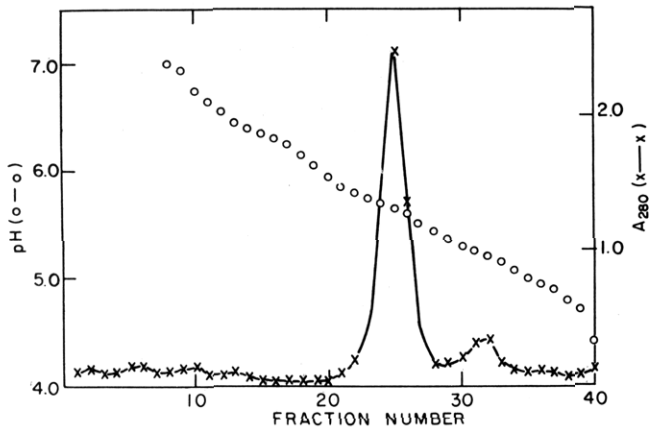


FIGURE 3: Isoelectric focusing of Fv. Electrofocusing of 10 mg of Fv, on the 110-ml ampholine column, was for 48 hr with 450 V. At the end of the run the column was drained at a flow rate of 20 ml/hr and fractions of 1.5 ml were collected.

and seems to be in accord with the molecular weight of Fv in sodium dodecyl sulfate (Inbar *et al.*, 1972), where it dissociates to two peptide chains. A comparative sodium dodecyl sulfate acrylamide electrophoresis of Fv, Fab, and protein 315 is shown in Figure 4. Fv shows only one component, Fab′ shows a fast band (L chain) and a slightly slower one (Fd, Underdown *et al.*, 1971), and protein 315 shows both light and heavy chains. The molecular weight of Fv in sodium dodecyl sulfate implies that it is composed of two peptide chains of similar size, each of them half the size of Fv.

This possibility also finds support in the N-terminal residues of Fv. The N-terminal residues of the light and heavy chains of protein 315 are PCA (Schulenberg *et al.*, 1971) and aspartic acid (by dansylation), respectively. The totally reduced and alkylated Fv fragment was subjected to pronase digestion (in 0.05 M $NH_4HCO_3$) and the digest was run on a Dowex 50X-2 column equilibrated with water. The unadsorbed fraction (detected by absorbance at 220 nm) was hydrolyzed in 6 N HCl and analyzed on the amino acid analyzer. The analysis showed only glutamic acid and alanine ($Glu_{1.2}$, $Ala_{1.0}$), which corresponds to the N-terminal sequence, PCA-Ala, of the light chain (Schulenberg *et al.*, 1971). The yield of PCA-Ala was found to be 83%. In addition, N-terminal aspartic acid was found in Fv by the dansyl method. N-Terminal sequence analysis of Fv is given in Table I. The high yield of the residues recovered indicates that, in addition to the $NH_2$-blocked PCA, Fv contains another chain with an unblocked $NH_2$ terminus. Indeed, the sequence found (Table I) is identical with that of the N terminus of the heavy chain of protein 315 (Francis, S., Leslie, R. G. Q., Hood L., and Eisen, H. N., personal communication). It is important to mention that, in each of the steps of Edman degradation, only one residue was found, indicating that there are no internal splits in Fv. Thus, Fv contains the same N-terminal residues as protein 315 and must, therefore, be composed of the N-terminal portions of the light and heavy chains.

TABLE I: N-Terminal Sequence of Fv.[a]

| Position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid | Asp | Val | Glu | Leu | Glu | Glu | ? | Gly | Pro | Gly |
| nmol | 87 | 73 | 58 | 55 | 57 | 57 | 0 | 52 | 45 | 48 |

[a] Fv (150 nmol) was subjected to automatic Edman degradation for ten steps. The phenylthiohydantoin amino acids were hydrolyzed at 150° with 6 N HCl and the recovered amino acids were determined on the amino acid analyzer. No correction was made for recovery during the hydrolysis.

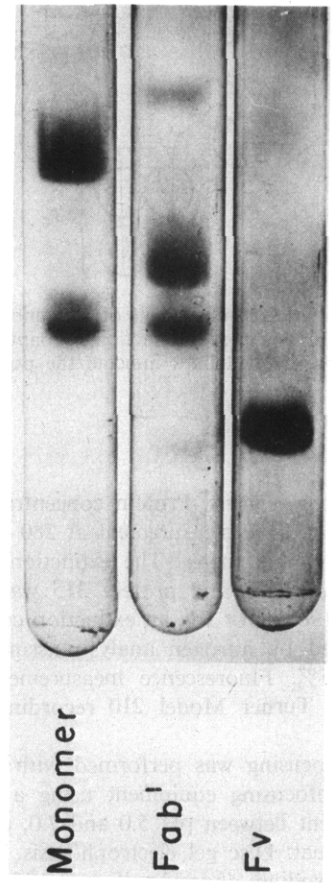


FIGURE 4: Disc electrophoresis of protein 315, Fab′, and Fv in 1% sodium dodecyl sulfate–10% polyacrylamide. Protein (50 μg) (reduced with 2-mercaptoethanol in 1% sodium dodecyl sulfate) was run in each gel employing 7 mA/tube.

*Separation of the Peptide Chains of Fv.* In view of the similar size of the peptide chains of Fv (Figure 4), we searched for their separation on the basis of charge difference. Cellulose acetate electrophoresis in 8 M urea, pH 9.0, showed a clear separation into two bands (Figure 5); therefore we chromatographed Fv on DEAE-cellulose under these conditions. In Figure 6 it is demonstrated that two fractions can be resolved by DEAE chromatography, each corresponding to one of the two bands in the cellulose acetate electrophoresis (Figure 5). The $A_{280}$ ratio of fraction I:II was 1:2, in good agreement with the tryptophan and tyrosine contents of the two components. Fraction I contains two Trp and two Tyr, whereas fraction II contains three Trp and eight Tyr (Table II). Using extinction coefficients of 5500 and 1360 for Trp and Tyr, respectively, the ratio is indeed 1:2.

Fraction I yielded only PCA-Ala (65% yield) and fraction II only aspartic acid as their N-terminal residues (Table III). This and the similarity of amino acid composition (Table II) between fraction I and that reported for the first 114 residues in the L chain (Schulenberb *et al.*, 1971) clearly demonstrate that fraction I is $V_L$ and fraction II is $V_H$. Other features of

TABLE II: Amino Acid Composition of Fv, $V_L$, and $V_H$.[a]

| Residue | Fv | $V_H$ | $V_L$ | $L_{1-114}$[b] |
|---|---|---|---|---|
| Lys | 8.6 | 4.3 | 4.4 | 4 |
| His | 2.9 | 1.0 | 2.2 | 2 |
| Arg | 7.5 | 3.1 | 4.4 | 4 |
| Asp | 22.5 | 15.1 | 8.0 | 7 |
| Thr | 27.6 | 12.5 | 14.2 | 16 |
| Ser | 19.3 | 12.1 | 8.1 | 9 |
| Glu | 18.0 | 11.4 | 8.6 | 9 |
| Pro | 9.5 | 3.6 | 4.3 | 5 |
| Gly | 23.0 | 11.0 | 14.0 | 14 |
| Ala | 13.0 | 3.4 | 10.2 | 10 |
| Half-Cys | 3.9 | 1.8 | 1.7 | 2 |
| Val | 14.9 | 7.0 | 7.5 | 9 |
| Met | 0.9 | 0.0 | 1.1 | 1 |
| Ile | 8.7 | 4.0 | 4.6 | 4 |
| Leu | 20.8 | 10.7 | 8.0 | 8 |
| Tyr | 10.2 | 8.2 | 1.9 | 2 |
| Phe | 10.8 | 5.8 | 5.4 | 6 |
| Trp[c] | 5.0 | 3.2 | 2.0 | 2 |
| Total | 227.1 | 118.2 | 110.7 | 114 |

[a] A known amount of protein, based on $A_{280}$, was hydrolyzed for 24 hr and applied to the amino acid analyzer. The $E^{280}_{1\%}$ of Fv, $V_H$, and $V_L$ were 15.0, 20.0, and 10.0, respectively. Recovery of residues per molecule was calculated on the basis of these data and the determined molecular weight of the proteins (Table III). [b] Taken from the sequence of the first 114 residues of the protein 315 L chain (Schulenberg *et al.*, 1971). [c] Determined by the spectroscopic method.

the amino acid composition of $V_L$ and $V_H$ are as follows: $V_L$ contains one methionine whereas no trace of Met was found in $V_H$. This can be used as one criteria for the separation of $V_L$ and $V_H$. In $V_H$ there are approximately 11 more acidic residues than in $V_L$, in accord with their charge difference (Figure 5). It is noteworthy that $V_L$ has four lysines and five prolines and this may give a clue to the position of pepsin split in $V_L$ (see Discussion).

The molecular weights of $V_L$ and $V_H$ were determined in 5.7 M Gdn·HCl. The results calculated from sedimentation equilibrium data as described above were 12,500 ± 600 and 12,300 ± 600 for $V_L$ and $V_H$, respectively.

Protein determination (by the Lowry method) on the two fractions obtained by DEAE-cellulose chromatography of Fv showed that they were obtained in a weight ratio of 1:1.1 ($V_L$:$V_H$).

These above results, summarized in Table III, provide evidence that Fv is composed of two peptide chains ($V_L$ and $V_H$) present in equimolar ratio. The amino acid composition (Table II), the presence of N-terminal residues of the parent light and heavy chains, and the molecular weight of the chains (in 5.7 M Gdn·HCl) indicate that the peptide chains of Fv are of the expected size for $V_L$ and $V_H$.

TABLE III: Properties of Fv and Its Peptide Chains.

| | Mol Wt | N-Terminal Residue | $E^{1\%}_{280}$ | Molar Ratio in Fv |
|---|---|---|---|---|
| Fv | 25,250 | PCA, Asp | 1.5 | |
| $V_L$ | 12,500 | PCA | 1.0 | 1 |
| $V_H$ | 12,300 | Asp | 2.0 | 1 |

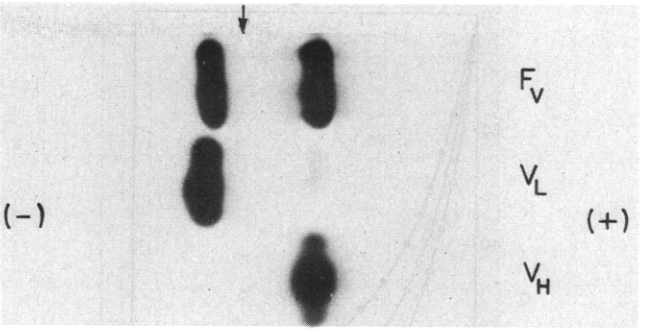


FIGURE 5: Cellulose acetate electrophoresis in 8 M urea of Fv and the fractions obtained from DEAE-cellulose (Figure 6). Electrophoresis was in 0.1 M Tris-HCl (pH 9.0)–8 M urea for 30 min at 250 V. Arrow indicates point of application.

*Binding Activity of Fv and the Recombined Chains.* Since Fv is devoid of the C-terminal halves of the peptide chains which compose Fab, it was of interest to compare the binding properties of the two fragments. Fluorescence quenching curves obtained by the addition of hapten to protein 315, Fab′ or Fv, are given in Figure 7. It is shown that the smaller the size of the fragment, the higher is the quenching by hapten of its tryptophan fluorescence, without a change in the number of sites per protein.

Separated $V_L$ and $V_H$ were used to test the possibility of their association to form an active Fv. An equimolar mixture of both peptide chains in 8 M urea, pH 9, was neutralized with an equal volume of 0.5 M sodium phosphate, pH 7, and diluted stepwise in the cold to 0.8 M urea with 0.15 M NaCl–0.01 M sodium phosphate to give a final $A_{280}$ of 0.10. Fluorescence quenching analysis was carried out on the recombined as well as on the separated chains diluted in the same manner. It is shown that, whereas $V_L$ or $V_H$ show only marginal binding activity, the recombined mixture of $V_L$ and $V_H$ regains 87% of the Fv activity (Figure 8). It is worth noting that when $V_L$

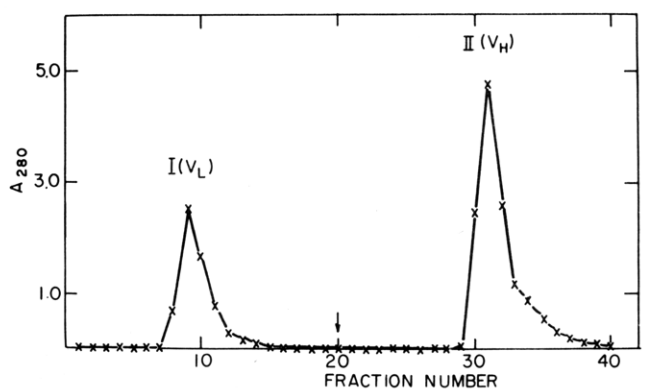


FIGURE 6: DEAE-cellulose chromatography in 8 M urea of Fv. The column was equilibrated with 0.1 M Tris-HCl (pH 9.0)–8 M urea, and 32 mg of Fv in the same solvent were applied. After the first fraction emerged from the column, 1.5 M NaCl–0.1 M Tris-HCl (pH 9.0)–8 M urea was applied (arrow). Column size was 1.4 × 17 cm and fractions of 2.5 ml were collected.

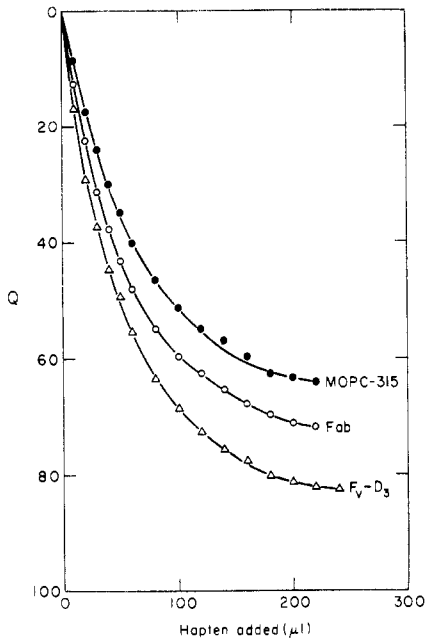


FIGURE 7: Fluorescence quenching titration of protein 315 monomer, Fab′, and Fv. Titration was with 50 μM $N^{\epsilon}$-Dnp-lysine at room temperature. Protein concentrations were $0.66 \times 10^{-6}$, $1.20 \times 10^{-6}$, and $1.28 \times 10^{-6}$ M in 0.15 M NaCl–0.01 M sodium phosphate, pH 7.4, for protein 315, Fab′, and Fv, respectively. Ordinate represents relative quenching (per cent).

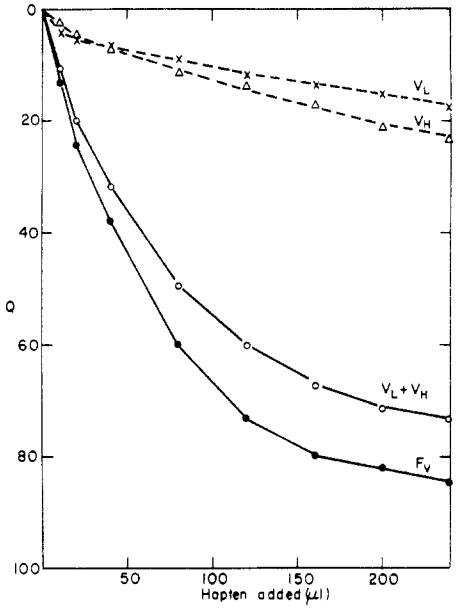


FIGURE 8: Fluorescence quenching titration of separated and recombined $V_L$ and $V_H$ derived from Fv. Protein concentrations were $6 \times 10^{-6}$, $4.2 \times 10^{-6}$, and $2.7 \times 10^{-6}$ M for $V_L$, $V_H$, and $V_H + V_L$, respectively. Protein solutions (except Fv) were in 0.15 M NaCl–0.01 M sodium phosphate– 0.8 M urea (pH 7.4). For conditions of $V_L$ and $V_H$ reassociation see text. Other conditions as in Figure 7.

and $V_H$ were separately diluted from 8 M urea into a buffer of a final urea concentration of 0.8 M, and then recombined in an equimolar ratio, a similar extent of activity was regained. Thus $V_L$ and $V_H$ can reassociate to form active Fv in either of the two ways shown in Scheme I.

## Discussion

The work presented in this article and in our previous one (Inbar *et al.*, 1972) is the first direct evidence for the localization of the antibody site in the N-terminal 110–120 residues of the antibody peptide chains. Indirect evidence suggested earlier that the antigen-binding site resides in this region; it is the only part of the sequence which varies from one antibody to another and can, therefore, provide the chemical basis for antibody specificity. Further, affinity labeling studies demonstrated that labeled residues were localized only in this region (Thorpe and Singer, 1970; Goetzl and Metzger, 1970; Franek, 1971; Haimovich *et al.*, 1972; Ray and Cebra, 1972). However, the demonstration that Fv has the same number of binding sites with the same affinity as Fab′ (Inbar *et al.*, 1972). provides evidence that, at least for protein 315, the constant region is neither necessary for the association between $V_L$ and $V_H$, nor does it provide an auxiliary effect to the properties of the binding site. It is very likely that the isolated Fv maintains its conformation as a globular unit, similar to that originally present in the intact molecule. This provides further support for the domain hypothesis. Table IV compares the various properties of Fv, Fab′, and protein 315.

Fv is composed of two peptide chains ($V_L$ and $V_H$) both of them starting at the N terminus of the parent chains (L and H). The amino acid composition (Table II) suggests that their size does not extend significantly beyond the size of the variable sequence in L and H. The amino acid composition of $V_L$ (Table II) indicates that it is a product of pepsin hydrolysis around position 115 in L. This is deduced from the following. (1) L contains three lysines up to residue 73 and a fourth one

SCHEME I

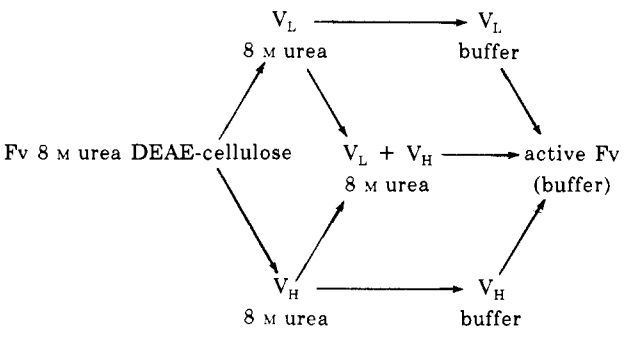


TABLE IV: A Comparison of Some Properties of Protein 315 and Its Fab′ and Fv Fragments.[a]

| | Protein 315 | Fab′ | Fv |
|---|---|---|---|
| $s_{20,w}$ | 7 S | 3.7 S | 26 S |
| Mol wt | 153,000 | 59,000 | 25,000 |
| Isoelectric point | pH 4.5 | pH 4.7 | pH 5.8 |
| Peptide chains | H, L | Fd, L | $V_H$, $V_L$ |
| N terminals | PCA, Asp | PCA, Asp | PCA, Asp |
| Binding sites | 2 | 1 | 1 |
| Association constant ($M^{-1}$) | $2.4 \times 10^6$ | $2.4 \times 10^6$ | $2.8 \times 10^6$ |

[a] The data are taken from this paper and from Inbar *et al.* (1972) and Eisen *et al.* (1968).

at 114 (Schulenberg *et al.*, 1971), $V_L$ contains four lysines, and, therefore, it is likely that $Lys_{114}$ is included in $V_L$. (2) L contains four prolines up to residue 61, an additional one around residue 110, and three more prolines between positions 117 and 123 (Schulenberg *et al.*, 1971). It is obvious that had the pepsin split occurred beyond position 123, $V_L$ would have eight proline residues. Since $V_L$ contains about five prolines and four lysines it is very likely that the pepsin split is somewhere between $Lys_{114}$ and $Pro_{117}$. Similar considerations for $V_H$ must await sequence data of the H chain of protein 315.

The molecular weight of $V_L$ and $V_H$ in 5.7 M Gdn·HCl or sodium dodecyl sulfate and the results of Edman degradation show that both chains are intact and without internal splits. The two chains are held together by noncovalent bonds and can be separated in 8 M urea. Upon dilution into buffer, $V_L$ and $V_H$ reassociate to yield an active Fv. The association constant between $V_L$ and $V_H$ must be greater than $10^6$ $M^{-1}$ since the affinity of Fv for Dnp-lysine, which requires that both chains will be in association, is greater than this value. Indeed, at a concentration of 10 $\mu$g/ml of $V_L$ and $V_H$, rapid recombination to form fully active Fv occurred.

It is obviously necessary to extend this finding to antibodies and myeloma proteins from other species. The possibility of obtaining such a small molecular weight fragment which contains all the structural requirements for the antibody binding site may prove to have been an important step in the fine structure analysis of antibody sites as well as in the preparation of synthetic antibodies.

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